Both Lipolysis and Hepatic Uptake of VLDL Are Impaired in Transgenic Mice Coexpressing Human Apolipoprotein E*3Leiden and Human Apolipoprotein C1

Miek C. Jong, Vivian E.H. Dahlmans, Patrick J.J. van Gorp, Marco L. Breuer, Marc J.T.M. Mol, André van der Zee, Rune R. Frants, Marten H. Hofker, Louis M. Havekes

Abstract Transgenic mice overexpressing human *APOE**3-Leiden are highly susceptible to diet-induced hyperlipoproteinemia and atherosclerosis due to a defect in hepatic uptake of remnant lipoproteins. In addition to the human *APOE**3Leiden gene, these mice carry the human *APOC1* gene (*APOE**3Leiden-*C1*). To investigate the possible effect of simultaneous expression of the human *APOC1* gene, we examined the phenotypic expression in these *APOE**3Leiden-*C1* mice in relation to transgenic mice expressing the *APOE**3Leiden gene without the *APOC1* gene (*APOE**3Leiden-HCR). *APOE**3Leiden-*C1* and *APOE**3Leiden-HCR mice had comparable liver expression for the *APOE**3Leiden transgene and high total cholesterol levels on a sucrose-based diet compared with control mice (4.3 and 4.3 versus 2.1 mmol/L). In addition, on this diet *APOE**3Leiden-*C1* mice displayed significantly higher serum triglyceride levels than *APOE**3Leiden-HCR mice and control mice (4.4 versus 0.6 and 0.2 mmol/L). Elevated triglyceride and cholesterol levels were mainly in the VLDL-sized lipoproteins. In vivo turnover studies with endogenously triglyceride-labeled VLDL showed a reduced VLDL triglyceride fractional catabolic rate for *APOE**3Leiden-*C1* and *APOE**3Leiden-HCR mice compared with control mice (3.5 and 11.0 versus 20.4 pools per hour). To study whether the difference in fractional catabolic rates between the two transgenic strains was due to an inhibiting effect of apoC1 on the extrahepatic lipolysis or hepatic-mediated uptake of VLDL, turnover experiments were performed in functionally hepatectomized mice. Strikingly, both *APOE**3Leiden-*C1* and *APOE**3Leiden-HCR mice showed a decreased lipolytic rate of VLDL triglyceride in the extrahepatic circulation compared with control mice (1.5 and 1.8 versus 6.3 pools per hour). We conclude that next to an impaired hepatic uptake, overexpression of the *APOE**3Leiden gene influences the extrahepatic lipolysis of VLDL triglycerides, whereas simultaneous overexpression of the *APOC1* gene leads to a further decrease in hepatic clearance of VLDL. (***Arterioscler Thromb Vasc Biol.* 1996;16:934-940.)**

Key Words • apolipoprotein C • apolipoprotein E • hyperlipoproteinemia • transgenic mice

In humans carrying the *APOE**3Leiden gene, the accumulation of chylomicron and VLDL remnant lipoproteins in the circulation is inherited in a dominant fashion.[1] The underlying mechanism is assumed to be a defect of this variant in binding to the hepatic lipoprotein receptors.[2] We have studied[3,4] the effect of the apoE*3Leiden protein in lipoprotein metabolism in more detail by using transgenic mice expressing the human *APOE**3Leiden gene. The high-expressing *APOE**3-Leiden transgenic mouse lines accumulate remnant lipoproteins in the plasma and develop atherosclerotic lesions, especially after consuming fat- and cholesterol-containing diets. As expected, in vivo VLDL-apoB turnover studies show that the hypercholesterolemia in these mice is indeed due to a defect in hepatic uptake of remnant lipoproteins.[5]

Since at the time of the generation of these mice the exact location of the HCR of the *APOE* gene was unknown, except that it lies about 9 kb downstream of the *APOC1* gene,[6] we used a 27-kb DNA construct covering the *APOE**3Leiden and *APOC1* genes and the HCR element. Western blot analysis showed that these *APOE**3-Leiden transgenic mice express both genes (designated *APOE**3Leiden-*C1* transgenics).[3] Thus, we cannot exclude that the hyperlipidemia and atherosclerosis observed in these transgenic mice is (partly) due to the coexpression of the human *APOC1* gene.

Little is known about the in vivo function of the apoC1 protein, although in vitro studies have shown that apoC1 can inhibit the LPL-mediated hydrolysis of VLDL TGs.[7,8] Studies involving the addition of purified human apoC1 to chylomicrons[9] and TG emulsions[10] show inhibition of their uptake by perfused rat livers. Furthermore, apoC1 prevents apoE-mediated VLDL binding to the LDL receptor[11] and the LDL receptor–related protein in vitro,[12,13] presumably by displacing apoE from the VLDL particle.[14]

The observation that transgenic mice with high liver-specific expression of the human *APOC1* gene exhibit hypertriglyceridemia[15] suggests that a direct inhibiting effect of apoC1 on either apoE-mediated remnant clearance or VLDL TG lipolysis holds true for the in vivo situation as well. However, such a role for apoC1 could not be deduced from the results we obtained with *ApoC1*-defi-

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From the TNO-Prevention and Health (M.C.J., V.E.H.D., L.M.H.), Gaubius Laboratory, Leiden; MGC-Department of Human Genetics (P.J.J. van G., A. van der Z., R.R.F., M.H.H.) and Medical Biochemistry (M.L.B.), Department of Molecular Carcinogenesis, Leiden University, Leiden; and the Department of Medicine (M.J.T.M.M.), Division of General Internal Medicine, University Hospital Nijmegen, Nijmegen, Netherlands.

Correspondence to Dr L.M. Havekes, TNO-PG, Gaubius Laboratory, PO Box 2215, 2301 CE Leiden, Netherlands. E-mail LM.HAVEKES@PG.TNO.NL.

Selected Abbreviations and Acronyms

FCR = fractional catabolic rate
FFA = free fatty acid
HCR = hepatic control region
HFC = high fat/high cholesterol
K_m = Michaelis-Menten constant
LFC = low fat/low cholesterol
LPL = lipoprotein lipase
LR = lipolytic rate
PAGE = polyacrylamide gel electrophoresis
SDS = sodium dodecyl sulfate
SR = secretion rate
SRM-A = standard rat mouse
TG = triglyceride
V_{max} = maximal enzyme activity

cient mice.[16] Although a lower level of plasma lipids was expected in these mice, we observed that mice with total apoC1 deficiency have normal plasma cholesterol levels on a regular chow diet, whereas after a severely hypercholesterolemic diet, *ApoC1*-deficient mice exhibit mildly elevated plasma cholesterol levels. Thus, the exact role apoC1 plays in remnant lipoprotein metabolism in vivo cannot be deduced from these studies with genetically modified mice.

In the present article we report on our study of the effect of *APOC1* gene coexpression on the phenotype of *APOE**3-Leiden transgenic mice by comparing the *APOE**3Leiden-*C1* mice described above with transgenic mice carrying only the *APOE**3Leiden gene directly linked to the HCR element, thus without coexpressing the *APOC1* gene (designated *APOE**3Leiden-HCR transgenics). By performing in vivo turnover studies with autologous TG-labeled VLDL samples, we found that additional apoC1 protein indeed inhibits the hepatic uptake of remnant lipoproteins in vivo, whereas apoC1 does not inhibit VLDL TG lipolysis. More strikingly, besides an inhibitory effect on hepatic uptake, we found that enrichment of VLDL particles with the apoE*3-Leiden protein also resulted in a disturbed extrahepatic lipolysis of VLDL TGs.

Methods

DNA Construct

The *APOE**3Leiden-HCR construct was generated from plasmid pJS276 (kindly provided by Dr J.D. Smith, Rockefeller University, New York, NY), which carries both the *APOE**3 gene (from the −650-bp *Bgl* II site to the 1.9-kb *Hin*dIII site) and a 5.5-kb *Bam*HI fragment from the 5′ region of *APOC1′*, including the HCR.[17] The *APOE**3Leiden gene was introduced into pJS276 by exchanging a 2-kb *Eco*RI fragment encompassing exon 4 of the *APOE**3 gene with the similar fragment from a cosmid carrying *APOE**3Leiden. The resulting insert (*APOE**3Leiden-HCR) was excised from the plasmid by using the restriction enzymes *Kpn* I and *Hin*dIII.

Generation and Analysis of Transgenic Mice

*APOE**3Leiden-*C1* mice were generated previously.[3,4] Transgenic and nontransgenic littermates were obtained by breeding with C57BL/6J mice. Mice of the F7 generation were used for the present experiments.

*APOE**3Leiden-HCR mice were generated according to the method of Hogan et al[18] with some minor modifications. In brief, a DNA solution was microinjected into male pronuclei of fertilized mouse eggs taken from superovulated F1 (C57BL/6J*CBA/J) females. Transgenic offspring were identified by polymerase chain reaction analysis and Southern blot analysis on genomic tail-derived DNA.[3] Six founders were obtained from which one line with high liver expression of the *APOE**3Leiden transgene was bred with C57BL/6J mice. Mice of the F4 generation were used for the experiments.

All mice in this study were females housed under standard conditions with free access to water and food. Nontransgenic littermates were used as a control group. All experiments were performed at 1 PM with food withdrawn at 9 AM.

Northern Blot Analysis

Transgenic mice were anesthetized with an intraperitoneal injection of 0.5 mL/kg Hypnorm (Janssen Pharmaceutical) and 12.5 mg/kg midazolam (Roche Netherlands bv), and the livers were excised for quantification of *APOE**3Leiden and *APOC1* mRNA. Total RNA was isolated from liver by using the RNAZOL procedure (Cinna/Biotecx). RNA samples (7.5 μg per lane) were separated by electrophoresis through a denaturing agarose gel (1% wt/vol) containing 7.5% formaldehyde and were transferred to a nylon membrane (Hybond N, Amersham) according to the manufacturer's recommendations. Blots were subsequently hybridized with ^{32}P-labeled probes of human *APOE* cDNA, human *APOC1* cDNA,[19] mouse *ApoC1* cDNA,[20] and a rat GAPDH cDNA[21] in a solution containing 50% formamide. The intensity of the hybridization signal was quantified by using a Phosphor Imager (Molecular Dynamics). The amounts of *APOE**3Leiden and *APOC1* mRNA were related to the level of GAPDH mRNA.

Diets and Dietary Treatment

After weaning at 28 days of age, mice were fed an SRM-A (chow) diet. After 7 weeks of age, two different diets (Hope Farms) were administered to groups of each strain (at least eight mice per group). These diets were semisynthetic and composed essentially according to Nishina et al.[22] First, mice were fed an LFC diet containing 50.5% sucrose, 12.2% corn starch, 5% corn oil, and 5% cellulose (by weight)[4] for 3 weeks. Thereafter, the same mice were fed an HFC diet for 3 weeks containing 15% cocoa butter, 0.25% cholesterol, and 40.5% sucrose (by weight).[23] After each dietary treatment, 100 μL whole blood was obtained from each fasting mouse via tail bleeding.

Analysis of Lipids and Lipoproteins

Levels of total serum cholesterol, serum TGs (without free glycerol), and FFAs were determined enzymatically by using commercially available kits (No. 236691 [Boehringer Mannheim GmbH], No. 337-B [Sigma GPO-Trinder kit], and a Nefa-C kit [Wako Chemicals GmbH], respectively).

For fast protein liquid chromatography fractionation of lipoproteins, 200 μL pooled serum from at least eight fasted mice per group was injected onto a 25-mL Superose 6 preparation-grade column (Pharmacia) and eluted at a constant flow rate of 0.5 mL/min with phosphate-buffered saline, pH 7.4. Fractions of 0.5 mL were collected and assayed for cholesterol and TG levels as described above.

Protein concentrations in lipoprotein fractions were determined by using the method of Lowry et al[24] with bovine serum albumin as a standard.

VLDL fractions (d<1.006 g/mL) were isolated from the pooled serum of at least 10 mice by ultracentrifugation at 40 000 rpm in an SW-40 swingout rotor (Beckman) for 18 hours at 5°C.

Quantification of Human ApoE

Serum human apoE*3Leiden concentrations were measured by using a sandwich enzyme-linked immunosorbent assay.[4] Affinity-purified polyclonal goat anti-human apoE antibodies were used for coating, and polyclonal rabbit anti-human apoE was used as the secondary antibody. After incubation of the plates with swine anti-rabbit IgG antibodies conjugated to horseradish peroxidase, detection was done by the immunoperoxidase procedure using tetramethylbenzidine as substrate. Pooled plasma from healthy human subjects with known apoE levels was used as a standard.

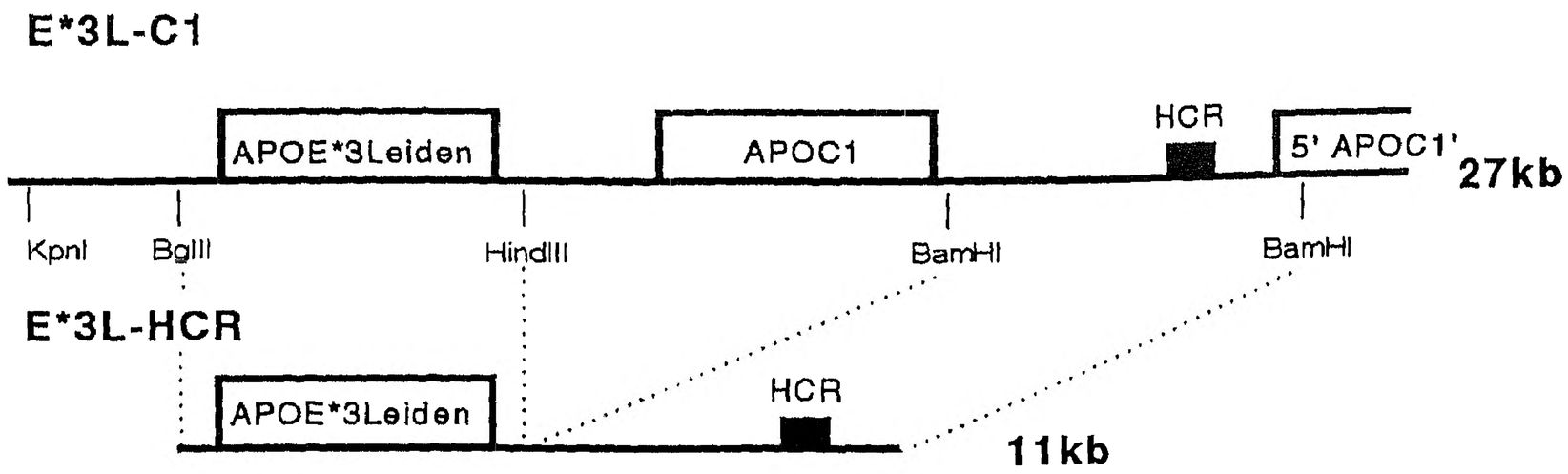

FIG 1. Schematic representation of the *APOE-APOC1* gene cluster. Top, 27-kb construct used for the *APOE**3Leiden-*C1* (E*3L-C1) transgenic mice. Bottom, 11-kb construct used for the *APOE**3Leiden-HCR (E*3L-HCR) transgenic mice. Open boxes indicate genes; black boxes, the HCR.

SDS-PAGE and Western Blot Analysis

From each lipoprotein fraction, 7.5-μg protein samples were analyzed for apolipoproteins by using SDS-PAGE with 4% to 20% gradient gels. Proteins were either stained with Serva blue R or transferred to nitrocellulose membranes (Schleicher and Schuell) followed by incubation with polyclonal rabbit antisera against mouse apoC1 or apoE and human apoC1 or apoE. Goat anti-rabbit IgG conjugated to peroxidase (Nordic Immunology) was used as the secondary antibody, and detection was done by using the immunoperoxidase procedure with 4-chloro-1-naphthol as substrate.

Preparation of Endogenously Labeled VLDL

Fasted mice were anesthetized with 0.5 mL/kg IP hypnorm (Janssen Pharmaceutica) and 12.5 mg/kg IP midazolam (Roche). Body temperature was maintained with the use of heat lamps. [^{3}H]palmitate dissolved in ethanol (Amersham) was evaporated under nitrogen and redissolved in 0.9% NaCl containing 2 mg/mL bovine serum albumin. Mice were injected intravenously via the tail vein with 100 μCi of the prepared [^{3}H]palmitate. To determine the efficiency of the in vivo [^{3}H]palmitate incorporation into VLDL TGs, 50-μL blood samples were drawn at 2, 10, 20, 30, and 60 minutes after the [^{3}H]palmitate injection. Lipids were extracted from the serum according to the method of Bligh and Dyer,[25] and the amount of radioactivity in the TG fraction was determined after separation of the TGs from the other lipid components by using thin-layer chromatography on silica gel 60 plates (Merck) with hexane/diethylether/acetic acid (83:16:1, vol/vol/vol) as resolving solution. [^{14}C]tripalmitate (Amersham) was used as an internal standard, and the proportion of the radioactivity in the plasma TG fraction was calculated in relation to the body mass of the mice.[26] To obtain VLDL radiolabeled in its TG moiety, anesthetized mice were injected as described above and bled from the retro-orbital plexus 25 minutes after injection. Radiolabeled VLDL (d<1.006 g/mL) used for clearance studies was isolated from the serum of six mice per group by using ultracentrifugation. These VLDL samples were dialyzed against phosphate-buffered saline, pH 7.4, at 4°C and subjected to lipid extraction and thin-layer chromatography analysis. In all the VLDL fractions used, >95% of the radioactive label was bound to TGs.

In Vivo Turnover Studies Using [^{3}H]TG-Labeled VLDL

Whole Animal

To study the in vivo clearance of labeled VLDL TGs due to both peripheral lipolysis and hepatic uptake of the remnant particle, fasted mice were anesthetized and injected intravenously with 80 000 dpm [^{3}H]TG-labeled VLDL (autologous injections). The disappearance of the radiolabeled VLDL was determined from 40-μL blood samples drawn at times after the injection as indicated. Total plasma radioactivity was used to represent VLDL TG radioactivity, as a pilot study showed that the disappearance of radioactivity as measured after lipid extraction followed by thin-layer chromatography TG analysis did not differ from the disappearance of total plasma radioactivity (not shown). The radioactivity at each time point was multiplied by the plasma volume of the animal[26] and divided by the injected dose. The data were modeled by a biexponential curve from which the FCR was calculated by using the reciprocal area under the curve. The SR was calculated by multiplying the FCR by the plasma VLDL TG pool size as measured in each mouse during the experiment.

Functionally Hepatectomized Animal

To investigate the in vivo clearance of radiolabeled VLDL due to peripheral lipolysis only, mice were functionally hepatectomized to exclude hepatic VLDL TG production and uptake.[27] Mice were anesthetized, and the hepatic portal vein and the hepatic artery were ligated prior to injections. [^{3}H]TG-labeled VLDL was injected, and blood samples were drawn and analyzed as described above. The data were kinetically modeled to calculate the LR by using the reciprocal area under the curve. To ensure total exclusion of the liver from the circulation, liver radioactivity was measured at the end of the experiment by using a sample oxidizer (Packard Instrument Co). For each hepatectomized mouse used in this study <0.5% of the injected dose was found in the liver.

In Vivo Hepatic VLDL TG Production by Triton WR1339 Injection

Fasted mice were injected with Triton WR1339 (500 mg/kg body wt IV) by using a 15% (wt/vol) Triton solution in 0.9% NaCl. Plasma VLDL clearance is virtually completely inhibited under these circumstances.[28] Blood samples were drawn at appropriate times (up to 60 minutes) after the Triton WR1339 injection. TGs were measured in the plasma and related to the body mass of the mice as described above. Production of hepatic TGs was calculated from the slope of the curve and expressed as millimoles per hour per kilogram body weight.

LPL-Mediated In Vitro Lipolysis of VLDL

In vitro lipolysis assays with isolated VLDL fractions (d< 1.006 g/mL) were performed at 37°C in a 0.1-mol/L Tris buffer, pH 8.5, for 10 minutes with commercially available LPL (Sigma) in the presence of 2% (wt/vol) essentially FFA-free albumin. The reaction was stopped by adding stop buffer containing 50 mmol/L KH_2PO_4 and 0.1% Triton X-100, pH 6.9, and placed on ice. To obtain a time zero control, the reaction was prevented by adding stop buffer prior to adding LPL and placed on ice. FFAs were measured as described above. The rate of FFA release by LPL was linear for the 10 minutes used in this assay. The assay was performed on five different VLDL concentrations (d<1.006 g/mL) in the range of 0.05 to 0.5 mmol/L with duplication of FFA determination. Apparent K_m and V_{max} of VLDL as substrate for LPL were calculated from Lineweaver-Burk plots.

Results

Characterization of *APOE**3Leiden-HCR and *APOE**3Leiden-*C1* Mice

Six founder mice carrying the *APOE**3Leiden-HCR construct (Fig 1) were generated. Two strains showed high-level expression in the liver. For the present study one of these strains was further characterized that showed hepatic *APOE**3Leiden mRNA levels comparable with the

previously generated *APOE**3Leiden-*C1* mice of line 2 (Table 1). In addition, *APOE**3Leiden-*C1* mice also exhibited hepatic expression of human *APOC1* mRNA,[3] whereas the hepatic mouse *ApoC1* mRNA levels of *APOE**3Leiden-HCR and *APOE**3Leiden-*C1* mice did not differ from that of control mice (99±25% and 109±26% of control value).

Human apoE*3Leiden levels, as quantified in individual mouse serum, were significantly elevated in *APOE**3Leiden-*C1* mice compared with *APOE**3Leiden-HCR mice (Table 1). The two transgenic mouse strains were further characterized by analyzing the apolipoprotein composition of VLDL fractions ($d<1.006$ g/mL). SDS-PAGE and Western blot analysis showed that both strains had equal amounts of human apoE*3Leiden protein on VLDL (Fig 2a). The amount of mouse apoE on VLDL was similar in both *APOE**3Leiden-HCR and *APOE**3Leiden-*C1* mice compared with control mice (Fig 2b). As expected, human apoC1 was found only on *APOE**3Leiden-*C1* VLDL (Fig 2c). Furthermore, mouse apoC1 (showing cross-reactivity with mouse apoC3) did not differ between *APOE**3Leiden-HCR, *APOE**3Leiden-*C1*, and control mice (Fig 2d). Serva blue R staining showed that the total amount of apoE was increased ≈twofold in both *APOE**3Leiden-HCR and *APOE**3Leiden-*C1* mice compared with control mice (Fig 2e). Analysis of the lipid composition of *APOE**3Leiden-HCR and *APOE**3Leiden-*C1* VLDL revealed no differences (not shown).

Serum lipids were measured in fasting transgenic mice and nontransgenic littermates as control animals. When kept on an SRM-A chow diet, both *APOE**3Leiden-*C1* and *APOE**3Leiden-HCR mice showed significantly elevated levels of serum cholesterol compared with control mice. Furthermore, serum TG levels were significantly elevated, being most pronounced in *APOE**3Leiden-*C1* mice (Table 1).

Feeding sucrose is known to stimulate hepatic VLDL TG production.[22] To investigate the response of both transgenic mouse lines to a sucrose-rich diet, mice were fed an LFC diet containing 50.5% sucrose. After 3 weeks on the LFC diet, the total serum cholesterol levels of both *APOE**3Leiden-*C1* and *APOE**3Leiden-HCR mice were elevated compared with their respective levels on the SRM-A diet and significantly different from control mice. In addition, upon sucrose feeding the serum TG level further increased in *APOE**3Leiden-*C1* mice, whereas, remarkably, it remained unaltered in *APOE**3Leiden-HCR mice (Table 1). This increase in serum TGs for *APOE**3Leiden-*C1* mice was confined to the VLDL-sized fractions as determined by fast protein liquid–Superose 6 chromatography (not shown).

FIG 2. Western blot analyses of mouse lipoproteins. VLDL ($d<1.006$ g/mL) was isolated by ultracentrifugation from the pooled serum of 9 control (lane 1), 9 *APOE**3Leiden-HCR (lane 2), and 9 *APOE**3Leiden-*C1* (lane 3) fasted mice. VLDL protein (7.5 μg) was subjected to SDS-PAGE (4% to 20% gradient gels) and transferred to a nitrocellulose membrane. The membrane was incubated with polyclonal antisera against human apoE (a), mouse apoE (b), human apoC1 (c), or mouse apoC1 (d). Total apoE was stained with Serva blue R (e). Note the double band in d, which is due to cross-reactivity of the mouse apoC1 antiserum with mouse apoC3.

We also compared both transgenic lines and control mice regarding their response to cholesterol feeding. After 3 weeks on the HFC diet (0.25% cholesterol), total serum cholesterol levels were increased among all groups compared with the LFC and SRM-A diets but were more pronounced in the transgenic mice. Serum TG levels in the *APOE**3Leiden-*C1* mice were lower on the HFC diet than on the SRM-A and LFC diets.[4] TG levels in *APOE**3Leiden-HCR and control mice remained unchanged upon cholesterol feeding (Table 1).

TG Turnover Studies in *APOE**3Leiden Transgenic and Control Mice

To investigate the mechanisms underlying the pronounced hypertriglyceridemia in *APOE**3Leiden-*C1* mice relative to *APOE**3Leiden-HCR and control mice, we performed VLDL TG turnover studies. Prior to these experiments, all mice were fed for 3 weeks with the sucrose-containing LFC diet because the hypertriglyceridemia of *APOE**3Leiden-*C1* mice was most pronounced when fed this diet.

TABLE 1. Characterization of *APOE3Leiden and Control Mice**

Mouse Strain	ApoE*3Leiden		Diet					
			SRM-A		LFC		HFC	
	mRNA in Liver, %	Protein in Serum, mg/dL	TC	TG	TC	TG	TC	TG
E*3L-C1	100±30	53±13†	3.0±0.6*	1.9±0.6*†	4.3±0.9*	4.4±1.1*†	8.5±1.1*†	1.3±0.7*
E*3L-HCR	110±25	35±11	2.8±0.6*	0.8±0.3*	4.3±0.6*	0.6±0.2*	6.8±0.6*	0.6±0.4*
Control	ND	ND	1.8±0.1	0.4±0.1	2.1±0.4	0.2±0.2	2.8±0.3	0.2±0.1

mRNA concentrations are relative to an internal standard GAPDH and are expressed as a percentage of *APOE**3Leiden-*C1* mice. Human apoE*3Leiden was quantified in mouse serum by using a sandwich enzyme-linked immunosorbent assay. Values for mRNA and protein are from mice fed SRM-A chow. Total cholesterol (TC) and TG values are expressed in millimoles per liter and were measured in serum of *APOE**3Leiden and control mice fed an SRM-A chow, LFC, or HFC diet for 3 weeks. Values for all measurements are mean±SD (n=8 mice/group). *$P<.05$ control vs transgenic animals on the same diet; †$P<.05$ *APOE**3Leiden-*C1* (E*3L-C1) vs *APOE**3Leiden-HCR (E*3L-HCR) on the same diet by nonparametric Mann-Whitney *U* test. ND indicates not detectable.

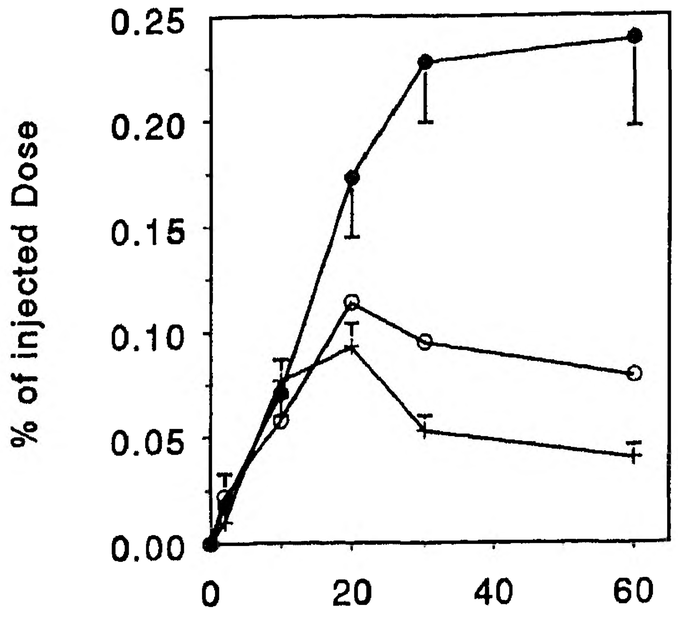

FIG 3. Line graph shows TG turnover studies in *APOE**3Leiden-*C1* (●), *APOE**3Leiden-HCR (○), and control (+) mice. Mice fed the LFC diet for 3 weeks were fasted and injected intravenously with 100 μCi [^{3}H]palmitate. The appearance of the label in plasma TGs over time was expressed as a percentage of the injected dose (see "Methods"). Each point represents mean±SD for six mice per group.

*APOE**3Leiden-*C1*, *APOE**3Leiden-HCR, and control mice were injected with [^{3}H]palmitate, and the appearance of label in plasma TGs was followed over time. Almost all the [^{3}H]palmitate (99.4%) was cleared from the plasma for all groups 2 minutes after injection (results not shown). The initial rate of appearance in the plasma of ^{3}H-labeled TGs was similar for both transgenic and control mice (Fig 3). However, the larger area under the curve suggests that clearance from the plasma was delayed in *APOE**3Leiden-*C1* mice compared with *APOE**3Leiden-HCR and control mice.

Endogenously labeled VLDL (d<1.006 g/mL) was isolated from serum collected from mice 25 minutes after [^{3}H]palmitate injection. After autologous injection, the labeled VLDL TGs were cleared at a reduced rate in *APOE**3Leiden-*C1* mice compared with *APOE**3Leiden-HCR and control mice (34% versus 14% and 7% of the injected dose was still present in the plasma 12.5 minutes after injection; Fig 4). From these data the FCRs and SRs were calculated (Table 2). In *APOE**3Leiden-*C1* and *APOE**3Leiden-HCR mice the VLDL FCRs were significantly decreased compared with that in control mice (3.5 and 11.0 versus 20.4 pools per hour).

TABLE 2. VLDL TG FCRs, SRs, and LRs for *APOE3Leiden and Control Mice**

Mouse Strain	FCR, pool/h	SR, mmol·h^{-1}·kg^{-1}	LR, pool/h
E*3L-C1	3.5±0.3*†	0.28±0.07*	1.5±0.9*
E*3L-HCR	11.0±1.8*	0.26±0.08*	1.8±1.1*
Control	20.4±3.2	0.17±0.04	6.3±2.1

FCRs were calculated from in vivo clearance studies of labeled autologous VLDL in transgenic and control mice (Fig 4). From these data SRs were calculated by multiplying the FCR by the pool size. LRs were calculated from the in vivo clearance of labeled VLDL in hepatectomized transgenic and control mice (Fig 5). Values are mean±SD (n=7 mice/group). *P<.05 control vs transgenic mice; †P<.05 *APOE**3Leiden-*C1* (E*3L-C1) vs *APOE**3Leiden-HCR (E*3L-HCR) by nonparametric Mann-Whitney U test.

VLDL SRs were significantly elevated for both *APOE**3Leiden-*C1* and *APOE**3Leiden-HCR mice compared with control mice (Table 2). Direct assessment of the in vivo VLDL TG production rate by injecting Triton WR1339 gave similar results (0.23±0.10, 0.17±0.07, and 0.14±0.08 mmol·h^{-1}·kg^{-1} for *APOE**3Leiden-HCR, *APOE**3Leiden-*C1*, and control mice, respectively). These differences in VLDL production rate were not highly significant and can only partly explain the strongly decreased FCRs observed in both transgenic mouse lines.

The VLDL TG FCR in *APOE**3Leiden-*C1* mice was much lower than that in *APOE**3Leiden-HCR mice (3.5 versus 11.0 pools per hour). A disturbed extrahepatic LPL-mediated lipolysis in the *APOE**3Leiden-*C1* mice could explain this difference. To test this hypothesis, control and transgenic mice were functionally hepatectomized to rule out VLDL TG clearance via the liver lipoprotein receptors. The rate of clearance from the extrahepatic circulation of [^{3}H]TG-labeled autologous VLDL was hampered in both transgenic lines compared with control mice (Fig 5). By calculating the LR from these experiments, we could observe no significant difference between the two transgenic

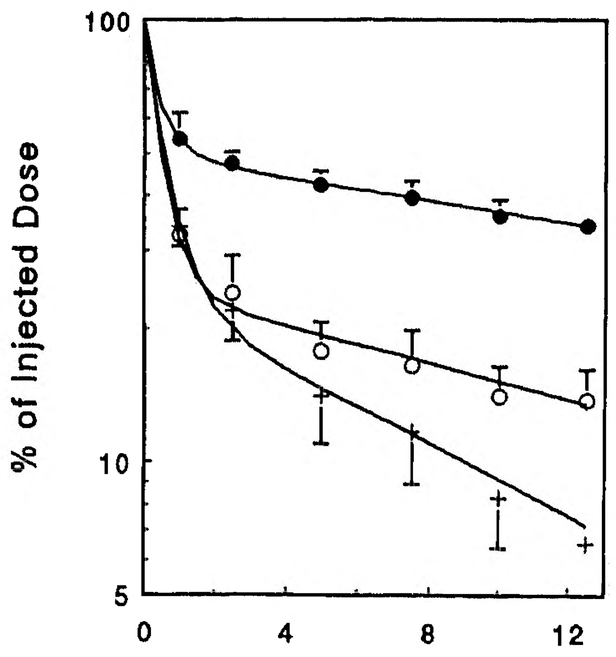

FIG 4. Line graph shows clearance of labeled VLDL in *APOE**3Leiden-*C1* (●), *APOE**3Leiden-HCR (○), and control (+) mice. Mice fed the LFC diet for 3 weeks were fasted and injected intravenously with 80 000 dpm in vivo labeled autologous VLDL. The disappearance of the labeled VLDL was followed by counting the plasma radioactivity expressed as the percentage of the injected dose. Values are mean±SD for seven mice per group. Curves were calculated from the mean data by using a biexponential curve fit model.

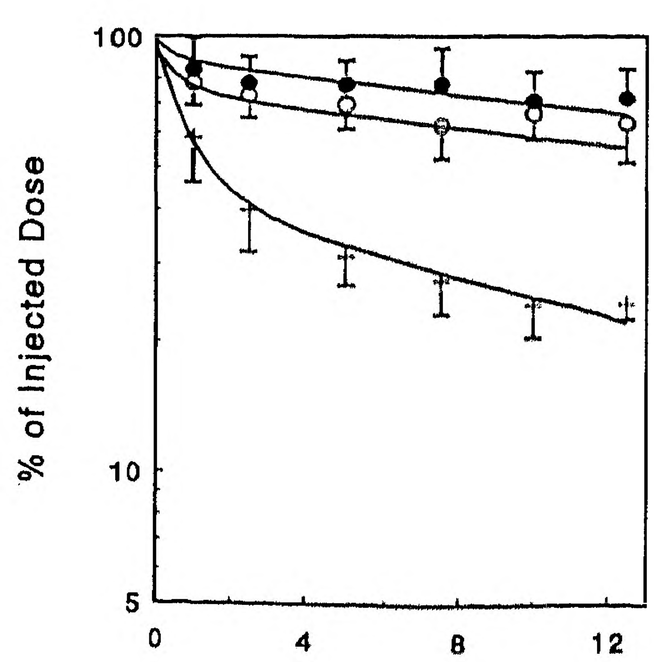

FIG 5. Line graph shows clearance of labeled VLDL in *APOE**3Leiden-*C1* (●), *APOE**3Leiden-HCR (○), and control (+) mice. Mice fed the LFC diet for 3 weeks were functionally hepatectomized, and 80 000 dpm in vivo labeled autologous VLDL was injected. The disappearance of the labeled VLDL was followed by counting the plasma radioactivity expressed as the percentage of the injected dose. Values are mean±SD for seven mice per group. Curves were calculated from the mean data by using a biexponential curve fit model.

mouse strains. However, the LRs of both *APOE**3Leiden-HCR and *APOE**3Leiden-*C1* mice were significantly lower than that of control mice (1.8 and 1.5 versus 6.3 pools per hour; Table 2).

These results indicate that the presence of human apoE*3Leiden on the VLDL particle inhibits the lipolysis of VLDL TGs in the extrahepatic circulation in vivo. However, this is not true for in vitro lipolysis. Lineweaver-Burk plot analysis with VLDL samples ($d<1.006$ g/mL) isolated by ultracentrifugation from pooled serum from *APOE**3Leiden-*C1*, *APOE**3Leiden-HCR, and control mice revealed no differences among the different VLDL samples in suitability as substrate for LPL in vitro. No significant differences could be observed between the respective VLDL samples in both the apparent K_m and V_{max} values (Table 3).

TABLE 3. Apparent Kinetic Parameters of VLDL for LPL-Mediated Lipolysis In Vitro

VLDL	K_m, mmol/L TG	V_{max}, mmol·L FFA^{-1}·min/U LPL^{-1}
E*3L-C1	0.65±0.26	1.00±0.49
E*3L-HCR	0.40±0.05	1.09±0.39
Control	0.59±0.30	0.45±0.17

Values for apparent kinetic parameters for serum VLDL preparations ($d<1.006$ g/mL) of *APOE**3Leiden-*C1* (E*3L-C1), *APOE**3Leiden-HCR (E*3L-HCR), and control mice were calculated from Lineweaver-Burk plots and are mean±SD of four independent experiments per VLDL sample.

Discussion

From our previous findings with *APOE**3Leiden transgenic mice we cannot definitely conclude that the observed hyperlipidemia and atherosclerosis in these mice is due exclusively to the expression of the *APOE**3Leiden gene, since these mice also express the human *APOC1* gene. In vitro studies suggest that the function of the apoC1 protein is related to LPL-mediated lipolysis in the extrahepatic circulation.[7,8] However, apoC1 may also inhibit the receptor-mediated uptake of VLDL remnants by the liver.[9-13] The recent finding that *APOC1* transgenic mice display hypertriglyceridemia[15] sustains these in vitro data but does not discriminate between the suggested functions for apoC1, since in these studies in vivo VLDL TG or VLDL apoB turnover studies were not performed.

We generated *ApoC1*-deficient mice to clarify the in vivo role of apoC1.[16] However, the data obtained with these mice did not accord with an inhibitory effect of apoC1 on VLDL catabolism. Although lower plasma lipid levels were expected in *ApoC1*-deficient mice, no overt effect of apoC1 deficiency on plasma lipid levels could be observed. We reasoned that the low plasma lipid levels usually found in normal mice, which are due to a highly efficient lipoprotein metabolism, are already too low to be further decreased. We therefore decided to study the role of apoC1 under conditions of hyperlipidemia in the *APOE**3Leiden mice by comparing the formerly described *APOE**3Leiden-*C1* mice with transgenic mice carrying only the *APOE**3Leiden gene directly linked to the HCR element. The generation of the *APOE**3Leiden-HCR DNA construct was made possible by the recent isolation of the HCR element by Shachter et al.[17] To discriminate between an effect of apoC1 on either extrahepatic lipolysis or hepatic uptake of VLDL, we performed in vivo turnover studies using autologous TG-labeled VLDL samples.

In both *APOE**3Leiden-*C1* and *APOE**3Leiden-HCR transgenics the FCR was significantly lower than in the control animals. For both transgenic lines the lower FCR can only partly be explained by a slightly increased VLDL production rate (Table 2). It is obvious from these VLDL turnover studies that the actions of apoE*3Leiden and apoC1 in inhibiting the overall in vivo VLDL TG clearance rate are additive. By performing turnover studies in functionally hepatectomized animals we were able to show that overexpression of the *APOC1* gene in addition to the *APOE**3Leiden gene indeed leads to a further inhibition of the hepatic uptake of remnant lipoproteins, since apoC1 does not affect in vivo lipolysis.

In vitro experiments[9-11] suggest that the inhibitory effect of apoC1 on the hepatic uptake of VLDL remnants is independent of the amount of apoE present on the particle but possibly due to an effect of apoC1 on the conformation of apoE. Other studies suggest that apoC1 displaces apoE from the particle, thereby decreasing the affinity of the particle for the receptor.[12-14] From our results a displacement of apoE by apoC1 is not likely to occur (Fig 2).

We have recently generated transgenic mice overexpressing the human *APOC1* gene only. Preliminary results show that the F1 generation of these *APOC1* mice are also hypertriglyceridemic due to a decreased VLDL TG FCR, whereas the in vivo lipolysis and production rate are not affected (unpublished results). Thus, these results support our conclusion that overexpression of *APOC1* rather than in vivo lipolysis inhibits hepatic uptake of VLDL remnants.

Transgenic mice overexpressing the human *APOC2* or *APOC3* gene are also hypertriglyceridemic.[28,29] In *APOC3* transgenic mice the accumulation of VLDL TGs may be due mainly to a decreased ratio of apoE/apoC3 on the VLDL particle, leading to a decreased apoE-mediated hepatic uptake of VLDL. In *APOC2* transgenic mice the relative amount of apoE on the VLDL particle is also decreased. As VLDL from these *APOC2* transgenic mice is less efficient in binding to heparin-Sepharose, it has been suggested that in *APOC2* transgenic mice VLDL particles are less accessible to cell surface–bound LPL, thus leading to an inefficient in vivo lipolysis.[30]

In addition to an inhibiting effect of apoC1 on the hepatic uptake of VLDL remnants, we also conclude from our results that enrichment of VLDL particles with the apoE*3Leiden protein results in a disturbed in vivo lipolysis of VLDL TGs. ApoE has an inhibitory effect on the LPL catalytic activity.[31] Furthermore, a synthetic peptide (residues 139 through 153) corresponding to the receptor-binding domain of apoE also inhibits LPL activity.[32] Although apoE*3Leiden differs from apoE by an additional repeat of residues 120 through 126,[3] it might still show the inhibitory action similar to that described for the synthetic peptide with residues 139 through 153.

Another explanation for the inhibiting effect of apoE*3Leiden on in vivo lipolysis might be that apoE*3Leiden containing β-VLDL displays decreased binding affinity to heparan sulfate proteoglycans.[33] A decreased interaction of apoE*3Leiden VLDL with the extracellular matrix of endothelial cells would imply a decreased accessibility of apoE*3Leiden VLDL to the LPL enzyme residing on this matrix, thus resulting in a disturbed in vivo lipolysis of apoE*3Leiden VLDL TGs.

The finding that both isolated apoE*3Leiden VLDLs are strongly enriched in apoE protein but do not differ from control VLDL in apparent K_m value for in vitro LPL lipolysis (using LPL in solution; Table 3) strongly argues for the

inability of apoE*3Leiden to bind to proteoglycans as the direct cause for the inhibiting effect of the excess of this protein on in vivo lipolysis. These results also indicate that future studies concerning the suitability of VLDL samples as substrate for LPL should be performed in a system in which the LPL enzyme is immobilized on, eg, a heparin-Sepharose column as described by Clark and Quarfordt.[34] This will resemble the in vivo situation of VLDL TG lipolysis much more than the system that is reported in Table 3. Experiments with immobilized LPL are currently being performed with the present VLDL samples.

In conclusion, we showed that excess apoC1 on the VLDL particle in vivo leads to a further impaired hepatic uptake of these particles from the circulation of *APOE**3Leiden-*C1* transgenic mice. In addition, an excess of apoE*3Leiden but not apoC1 on the VLDL particle hampers the in vivo lipolysis of VLDL TGs. Thus, the absolute and relative amounts of both apoE and apoC1 on VLDL particles might be strong factors for the underlying metabolic defect in hypertriglyceridemia.

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